Bioenergy Production Using *Trichormus variabilis* - A review

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Abstract

Fossil fuel processing and consumption contaminates air, soil, and water resources via release of hazardous chemicals. To protect the environment, harnessing renewable energy resources and development of sustainable technologies are prime targets of research and increased investment. Use of bio-based feedstocks in energy production inherently provides a valuable pollution-curbing pathway with sustainability credentials, especially when wastewater is used to provide the nutrient requirements. The filamentous cyanobacterium, *Trichormus variabilis*, has attracted substantial attention from researchers due to its potential for dual industrial functions in bioenergy production and bioremediation. This species can efficiently use the power of sunlight energy to fix atmospheric CO_2 for generating valuable chemical compounds, such as carbohydrates and fatty acids, which can be converted to biofuels. Because it grows in nutrient-rich wastewater (industrial effluent), it can serve as a bio-absorbent and replace costly chemical catalysts and nano-materials classically used for removal of nutrients and metals. However, no recent review has presented potential for state-of-the-art *T. variabilis*-driven phycoremediation-bioenergy production systems. Therefore, in order to present possible routes from phycoremediation to energy production as a strategy for developing the industrial application of *T. variabilis*, we present this review to bring important research results on this species together and highlight major related challenges and opportunities. The current status of applying algae in bioremediation and production of liquid and gaseous fuels utilizing wildtype and mutants of *T. variabilis* is explored. Finally, key points underlying potential for future research on optimization of robust technologies for supplying sustainable bioenergy using this organism are presented.

Key words: Bioenergy, Hydrogen, Bioremediation, *Trichormus variabilis*.

1 Introduction

Global economic growth over the last two centuries has largely been driven by increased use of fossil fuels and was accompanied by significant environmental pollution, particularly increased CO_2 emissions. Total world energy consumption is forecast to increase by 1.4% per annum up to 2040[1], exacerbating negative environmental impacts and increasing pressure on finite fossil fuel resources. Additionally, both economic growth and rises in the global population are reciprocated by increased demand for freshwater required for industrial processes, agriculture, and domestic use. Therefore, recycling wastewater becomes an imperative for water conservation. There exist a number of routes to transform the environmental challenges posed by wastewater into efficient, economically viable methods for both energy production and water recycling. Phycoremediation has been used since circa 1963 as a cost-effective nutrient removal method using microalgae to treat municipal wastewater[2]. In addition to CO_2 capture, phycoremediation has several other benefits, including removal, transformation, or degradation of nutrients, organic matter, acids, metals and xenobiotic compounds, and the use of algae as an environmental monitoring system for detection of toxic materials[3] and production of high-value metabolites[4]. Furthermore, massive biomass grown in wastewater streams can be used effectively as a promising source of bio-fuels such as bioethanol, biodiesel, bio-hydrogen or biogas, which are generally known as the most carbon neutral liquid and gaseous fuels, depending on their generation technology.

Previous reviews have collated research on phycoremediation[5], bioenergy production[6, 7], biorefining[8] and algal photobioreactors (PBRs)[7]. However, to our knowledge, none of these reviews have been dedicated to a specific strain of algae. Among many microalgae species, *Trichormus variabilis* has been studied extensively owing to its dual benefits: showing high potential in industrial and municipal wastewater treatment[9-11] and also its capability for bio-hydrogen production[12, 13]. The worldwide distribution of *T. variabilis*, high auto-flocculation capacity[3], tolerance of a wide temperature range, and bio-fertilizer potential[14] strongly affirm that the study of this species needs to be expanded in the future. Here, we present a comprehensive review, focused on *T. variabilis*, and its principal enzymatic functions which make it suitable for applications in biohydrogen production. Subsequently, we present the physico-chemical parameters affecting the morphology, physiology and growth of *T. variabilis* and evaluate how these factors might impinge on the hydrogen production process. Finally challenges and opportunities in developing a route to industrial application of *T. variabilis* as a green chemistry workhorse in effluent treatment and production of sustainable bioenergy are discussed.

2 Methodology

Primary and secondary literature and data used in synthesis of this review were obtained via searching Web of Science (https://clarivate.com/products/web-of-science/) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) databases and several sources of grey literature using keywords related to *T. variabilis* in terms of various types of bioenergy and nutrient removal potential. Synonyms of *T. variabilis*, such as *Anabaena variabilis*, were also used as keywords given that authors tend to use different names for this organism. Information was gathered in a two-phased process. In the first phase, inclusion criteria were peer-reviewed academic research and published industrial pilot studies or full-scale production systems. Because of paucity of published data specific

to *T. variabilis* no exclusion criteria were applied, except the exclusion of self-published, non-peer-reviewed material. In order to capture important data from grey literature, such as patents and technical reports, the second phase reversed the inclusion and exclusion criteria of the first phase. The taxonomic classification of *Trichormus variabilis* was extracted from data presented in major biological, botanical and algal databanks including AlgaeBase[15], World Register of Marine Species (WoRMS ID: 146661)[16] and National Center for Biotechnology Information (NCBI taxonomy ID: 240292, https://www.ncbi.nlm.nih.gov/). Genes encoding hydrogen biosynthetic enzymes (described in Section 6.3) were identified via literature and Genome Database Resources of CyanoBase in Kazusa DNA Research Institute (http://genome.microbedb.jp/cyanobase/). Vector graphics were drawn by Inkscape software v0.92[17]. To provide images of *T. variabilis* cells, algal samples were cultivated and processed for transmission electron microscopy (TEM) as described previously[18]. All the tables in this review were constructed from the primary results reported in different studies cited within each tables. Figure 1 presents a workflow chart illustrating the search strategy used.

Figure1. Flowchart of the research strategy. To develop each section of this literature review, a question was formulated and related keywords generated for interrogation of the databases. The returned information hit list was divided into three broad categories of algal cultivation for biomass production, algal cultivation for bioenergy production, and algal cultivation for phycoremediation. Progress of innovation across these were critically analysed by comparative analysis and current challenges and opportunities identified. Areas of future research to ensure current investments lead to commercial feasibility were finally identified and discussed.

3 *Trichormus variabilis*

Trichormus variabilis (*syn Anabaena variabilis* ATCC 29413) is a filamentous cyanobacterium from Nostocoidae subfamily[19, 20] found in both freshwater and within soil habitats. The recorded history of study of this species begins nearly two centuries ago, when it was collected for the first time in 1839 from puddles and drains in Hooksiel and Jadebusen, located in Wangerooge Island in Lower Saxony of Germany by Kutzing[21]. Since then, its widespread distribution has been reported across almost all continents, being a native species in many countries, from Asia to America, Africa and Australia[22, 23]. The taxonomy of this species has faced repeated revision owing to the formation of two main cell types (akinetes and heterocysts), including differentiation from the genus of *Nostoc*[24], *Wollea*[25] and *Anabaena*[19, 20, 24, 25] on the basis of akinete formation. According to the last rectified taxonomy (Fig. 2), it has been transferred from *Anabaena* to the genus *Trichormus*, with the former scientific name "*Anabaena variabilis*" proposed by Bornet and Flahault in 1886 being replaced with "*Trichormus variabilis*" as proposed by Komarek and Anagnostidis in 1989[25-27].

Figure 2. Taxonomy of *Trichormus variabilis*.

3.1 Morphology and Physiology

The filaments of *T. variabilis* are morphologically variable[19, 24]. Filaments mainly contain chains of barrel-shaped gram-negative vegetative cells (Fig. 3a) as a site for

photosynthesis with conical terminal cells (Fig. 3b) covered by a thin gelatinous polysaccharide sheath (Fig. 3c). The mucilaginous sheath encapsulating the filaments arises from polysaccharide secretions by the cells[28, 29]. Vegetative cells have a primary role of harnessing solar energy for fixing carbon to drive biomass production. The growth in biomass can be coupled to wastewater treatment through removal of heavy metal ions and nutrients. The most important cell differentiation in *T. variabilis* is the formation of heterocysts, also known as heterocytes. When transferred to diazotrophic conditions, some vegetative cells differentiate into heterocysts as a site for nitrogen fixation[25]. The spherical, ellipsoidal or cylindrical shape[24], the large size[25], distinctive polar bodies[30], and hollow appearance, make heterocysts readily recognisable among other cell types (Fig. 3d). Even though heterocysts represent only 5-10% of cells along the filaments[31], phylogenetic studies showed that about 45% of the genome is expressed in these cells[32]. Cell differentiation is underpinned by changes in gene expression, which trigger biochemical and structural transformations, such as formation of thick multi-membranous glycolipid walls, arrest of photosynthesis and biosynthesis of the nitrogenase enzyme complex[33]. These modifications prepare heterocyst cells for the vital role of fixing atmospheric nitrogen with hydrogen gas as a by-product of the reactions. Because nitrogenase enzymes are sensitive to oxygen, heterocysts bereft of oxygenic photosynthesis provide an anaerobic environment to stabilise the N-fixation system in an otherwise aerobic organism. Heterocysts strongly attach to vegetative cells via cyanophycin polar nodule[30] (Fig. 3d).

In response to nutrient limitation or environmental stress such as prolonged darkness, dryness, freezing, and nutrient depletion[30, 34, 35], specialised akinete cells develop as resilient archetypal spores to ensure survival of the organism in harsh environmental conditions. Akinetes are oval to barrel-shaped cells with granules filled with glycogen and cyanophycin[36], which can easily be distinguished from vegetative cells by their larger size and thicker envelope (Fig. 3e). Their thick outer layer contains glycolipid and polysaccharides, making them more resistant to stressful conditions in comparison to vegetative cells and heterocysts[28]. Akinetes germinate and develop into new filaments when favourable growth conditions are restored. On germination, an akinete develops into a short filament which is structurally distinguishable from trichorms in having 5 to 15 vegetative cells and lacking heterocysts (Fig. 3f). These short filaments, known as hormogonia, later develop into mature trichorms possessing heterocysts. - Therefore, the diverse cell functions provide an attractive system for commercial development of an efficient low-cost biomass production during wastewater treatment process, which could be coupled to industrial bioenergy production. The morphological appearance of various cell types of *T. variabilis* is illustrated schematically in Fig. 3, with Fig. 4 showing actual images of the cells using transmission electron microscopy (TEM).

Figure 3. Schematic depiction of different cell types of *T. variabilis*. (a) Vegetative cells; **(b)** Terminal cell; **(c)** polysaccharide mucilaginous sheath; **(d)** Heterocysts, which posses: 1. Fibrous layer, 2. Laminated layers (internal layers), 3. Plasmodesmata, 4.Polar nodule, and 5.Thylakoids; **(e)** Akinete, which has: 6. sheath, 7. additional wall, 8. Cyanophycin granule, 9. Inner layer, and 10. Dense fibrillar layer; **(f)** Hormogonium; **(g)** Outer membrane; **(h)** Polyphosphate granules.

Figure 4. Microscopic images of *T. variabilis* cells. (a) Low power light microscope image of filaments in nitrogen-depleted growth medium showing vegetative

cells, heterocysts, and akinete. **(b)** Transmission electron micrograph of filaments encased by a gelatinous polysaccharide sheath (PC) with terminal cells (TC) at the end of the trichorms. **(c)** Transmission electron micrograph showing a detached mature vegetative cell showing peptidoglycan layer (PG), carboxysome (CS), polyphosphate granule(PS), cyanophycin granule (CP), thylakoid and attached phycobilisomes (Tl). Scale bars: **(a)** 50 μm; **(b)** 2 μm; **(c)** 500 nm.

4 Physico-chemical factors affect *T. variabilis* biomass production

Knowing key physico-chemical factors required for promoting high rates of biomass production is necessary for developing industrial-scale bioreactors for algal cultivation. Zho[37] summarized general microalgae cultivation strategies, abiotic, biotic and operational factors affecting algal growth. *T. variabilis* is capable of growing phototrophically, heterotrophically/chemotrophically in the dark with exogenous sugar, and also mixotrophically in light with exogenous sugar[31, 38, 39]. This variation in growth strategy makes it a potentially favourable microorganism for use in different light- and dark-driven bioenergy production processes. *T. variabilis* is a mild thermophilic cyanobacterium, which has photosynthetic activity in a broad temperature range (10- 35 °C) and light intensity (42- 562 $\mu mol.m^{-2}.s^{-1}$)[40]. It was demonstrated that the growth yield declined (0.1 g dry mass.g^{-1} CO_2) by increasing the initial CO_2 concentration from 4 to 18%[41]. Chemical nutrients, essential salts and metals directly influence different metabolic modes, which consequently affects bioenergy yield and remediation potential. The main growth media used for *T. variabilis* include blue-green (BG)[42], Allen and Arnon (AA)[43], 2-(N-Morpholino) ethane sulfonic acid-Volvox (MES)[44], Marin (MN)[45], ammonium mineral salt (AMS-1)[46] and Fogg's Medium[47]. Among these, BG and AA are the most commonly used media, specifically when *T. variabilis* is used as a source of bio-hydrogen. Higher biomass production was reported when grown in BG_{11} enriched with a nitrogen source[48]. In general, BG and AA media are based on molybdenum (Mo) and vanadium (V), respectively. These two metals are required for activation of two key enzymes (described in section 5.3), which are responsible for hydrogen generation. Investigating the effects of replacing Mo with V demonstrated that replacement of Mo with V leads to decreases in the growth yield[43] while it improves hydrogen generation yield[48] (section 5.4).

Experiments establishing *T. variabilis* growth profiles have classically used, as proxies for biomass accumulation, parameters such as photosynthetic potential/capacity[49] and cell density at specified wavelengths[50]. These have been applied in studying the effects of altering various physicochemical factors such as light and nutrient supply, on *T. variabilis* phototrophic[51, 52] or heterotrophic[39, 53] growth. While a large number of studies have investigated *T. variabilis* growth rate as a function of time, cell density or photosynthesis components[54, 55], only a few have directly surveyed the interrelation between multiple parameters. Such data can be used for modelling growth kinetics. In prior work, a kinetic model for growth of *T. variabilis* was developed based on the Monod model as a function of CO_2 and light intensity[56]. Predictive models are increasingly important as algal biomass generated from wastewater treatment process is being used for production of biofuels when scaling-up from laboratory-scale to industrial-scale cultures. Therefore, the model should capture key parameters affecting growth rate so that bioreactor designs are optimised for maximal productivity.

Additionally, this species has variable morphology based on the type of mechanical stimulation. It normally grows as filaments that attach longitudinally to neighbouring

filaments to form thalloid structures, but when it is grown with orbital shaking, thalli formation is prevented, with a homogenous suspension of short filaments emerging[43]. High tendency of filaments for sticking to the walls blocks light penetration, which causes problems when cultivating this species in photobioreactors. The membranous growth form may provide an opportunity in flocculation of biomass in open raceway ponds. This potential, which resolves one of the most important challenges of industrial algal harvesting technologies in biofuel production, requires further investigation.

5 Biomass production via phycoremediation

Providing sufficient water and nutrients, particularly low-cost nitrogen, is a challenge for large-scale microalgae cultivation for bioenergy[7]. However, nutrient-rich wastewater streams can provide most of the nutrients required for algal growth. In addition to utilising nitrate and phosphate, microalgae have the capacity for effective removal of heavy metals. Incorporation of metals into the biomass may pose downstream risks during future processing or utilisation of derivatives, depending on the uses of the biomass. Owing to their rapid growth rates and potential to remove heavy metals, various microorganisms have been used in phycoremediation of municipal[57], industrial[58] or agricultural[59] wastewaters. These microorganisms include (i) green algae; *Chlamydomonas reinhardtii* [60], *Chlorella sp.*[61], *Nannochloropsis oculata*[62], *Scenedesmus dimorphus*[59], (ii) blue-green algae; including *Synechocystis salina*[63], *Spirulina platensis*[64], *Phormidium bohneri*[65], *Tolypothrix ceytonica*[9], (iii) brown algae, including *Padina* sp.[66], *Laminaria hyperborean*[67], and (iv) red algae such as *Porphyridium cruentum*[68]. Among the cyanobacteria, *T. variabilis* has received considerable attention for use in bioremediation. Attraction to this species has partly been the potential for coupling wastewater treatment with production of bioenergy. The growth and remediation performance of *T. variabilis* in treating water contaminated with ammonium[69] and biosorption of heavy metals, such as Cr, Cd, Ni and Pb[70] has been demonstrated. Therefore, *T. variabilis* is already noted as an efficient microalgae for actual and potential treatment of mixed industrial and municipal wastewater[9]. Table 1 presents a synopsis of key studies on phycoremediation using *T. variabilis* and the maximum removal efficiency reported for each process.

A study on phenol compounds remediation illustrated notable nitrophenol compound removal efficiency from an artificial supplemented wastewater using this species[71]. However, since polyphenolic compounds produced by *Myriophyllum verticillatum* cause growth inhibition of *T. variabilis* cells[72], the feasibility and efficiency of *T. variabilis* remedial action on phenolic contaminants will require further research. It was also reported that nanomaterials, such as nano-titanium dioxide ($nTiO_2$), have negative effect on the intracellular structure of *T. variabilis*[73-75]. Furthermore, *T. variabilis* growth is impaired by some allelochemicals such as Harmane (1-methyl-β-carboline)[76] and FischerellinB[77] causing cell lysis and photosynthesis inhibition respectively. Microcystins have intense allelopathic inhibition on *T. variabilis* growth[78] and differentiation of heterocysts and akinetes[79]. The presence of such pollutants in wastewater streams affects nutrient removal efficiency by disrupting metabolic processes and consequently, disrupts bioenergy production pathways. Therefore, more research on detection of growth inhibiting pollutants, their effects, and ways to deal with them is required. Exposing *T. variabilis* to pollutants can gradually change gene expression and acclimate the organism for growth in previously toxic compounds. For example, it was demonstrated that repeated cultivation of this species in a medium with

high concentration of $Cu(NO_3)_2$ developed a Cu-resistant strain with broad resistance to Cd, Zn and Ni[49]. Thus, genomics and proteomics research could provide a clear understanding of the exact metabolic changes ubderpinning this adaptive change.

6 Bioenergy production using *T. variabilis*

Several studies focused on integration of wastewater treatment and bio-fuel production using microalgal systems[80-83]. After nutrient removal, the biomass produced can effectively be utilized as a resource for cost-effective bioenergy production. We review the application of *T. varibilis* in bioenergy production from the perspective of liquid (biodiesel, bio-ethanol) and gaseous (bio-hydrogen, bio-methane) bio-fuels. Applying microalgae systems for bioenergy production has several environmental advantages, such as reduction of pollutant emissions and soil erosion, and economic benefits such as tax credits and grants without competing with agricultural produce for freshwater and arable land[84, 85]. In addition to these general advantages, *T. variabilis* has enhanced capacity for auto-flocculation[3] which is one of the most important characteristics to decrease energy consumption during algae biomass harvesting. Also, nutrient removal and CO_2 bio-fixation potential together with interesting cell differentiations in forming heterocysts and akinetes, which provide suitable opportunities for N-fixation and growth in challenging environmental conditions, respectively, and availability of processed biomass as bio-fertilizer[14], suggest this species may present a viable solution for algae bioenergy production with valuable by-products.

6.1 Liquid bio-fuels: biodiesel and bioethanol

Ethical concerns about diversion of crops from the human food chain to fuel production have spurred the search for inedible crop oils and waste fats for use as a bio-fuel feedstock. Inedible oilseeds, such as Jatropha, Castor, and other indigenous crops[86, 87] which can grow on marginal land have emerged as potential candidates. However, the cost of land, labour and freshwater resources often make large-scale production uneconomical when set against the yields attained. Therefore, microalgae received greater attention and interest due to their capability to grow in wastewater. Since lipid content is the most important key factor in utilization of oleaginous microorganisms as a source of biodiesel production, considerable research focuses on increasing lipid yield. Although *T. variabilis* is not considered as an oleaginous species, previous reports indicate notable increases in lipid content after exposure to environmental stress or nutrient limitation. For example, it was demonstrated that lipid content of this alga in N-depleted Arnon medium at 30-35 ^oC under 12:12 h light/dark cycle was increased by ~ 2.5-fold (yield~10.5%)[88]. Furthermore, it has been reported that ultrasound at 200 W after 5 minutes increased lipid content of *T. variabilis* grown in BG_{11} medium at 25 ^oC under continuous illumination by 1.46-fold (yield~47%)[89]. Cellular stress triggered by ultrasonic treatment was responsible for increased oil production while a longer duration of treatment damaged the cells. Such stress or nutrient limitation leading to increased lipid yield was also observed in other species, including *Nannochloropsis sp.*[90], *Chlorella sp.*[91] and *Chlamydomonas reinhardtii*[92]. These useful results can be considered as a promising solution for promoting lipid accumulation in the cells and consequently enhance the biodiesel production yield. However this also requires accurate investigation on the profile of fatty acid methyl esters to match the biodiesel quality standards.

From a bio-ethanol perspective, *T. variabilis* has not been widely investigated. The main challenge in bio-ethanol production from this filamentous species is overcoming the polysaccharide loss during processing. Research has shown that drying the biomass using supercritical fluid followed by fermentation could increase the bio-ethanol yield (24.1%) about 2-fold comparing with using lyophilization process followed by fermentation (yield~13.6%)[93]. A further study has confirmed that applying supercritical fluid pre-treatment efficiently enhances the amount of ethanol from 1.25 $g.L^{-1}$ to 2.28 $g.L^{-1}$ [94]. Evidently, more research is required to fully investigate the liquid bio-fuel production potential of *T. variabilis*, especially when it is grown in wastewater.

6.2 Gaseous bio-fuels: bio-methane and bio-hydrogen

Biogas is the oldest form of bioenergy generated by means of methanogenic bacteria under anaerobic conditions containing ~50-70% bio-methane, which can be used as a heat, power or transportation fuel[95]. Several microalgae have been used to combine bio-methane production with biorefinery approaches, for example, *Chlamydomonas reinhardtii and Scenedesmus obliquus*[96]. There are some reports of *T. variabilis* biomass conversion into biogas under high temperature using anaerobic digesters without[97] or in combination with immobilising technology to enhance gas production[98]. Methane production yield was recorded at 450 $mL.g^{-1}$ biomass using immobilised methanogenic bacteria and *Rhodobacter capsulatus* on polymeric matrices in an anaerobic bioreactor[98]. In another research cumulative methane yield (64%) was recorded at ~4 $mmol.g^{-1}$ biomass when anaerobic cellulolytic substrate were used together with methanogenic *Archaea* from genera of *Methanoculleus* and *Methanosarcina*[97]. Biogas is generated via 4 successive stages: (i) hydrolysis of biopolymers to monomers, (ii) fermentation (acidogenesis) of amino acids and sugars to intermediary products, (iii) acetogenesis of intermediary products to acetate, CO_2 and hydrogen, and (iv) methanogenesis, which transforms acetate into methane. During anaerobic digestion, hydrolysis is known to be the rate limiting step which needs to be optimized via efficient pre-treatment technologies. Owing to the composition and structural features of microalgae resulting in changes in response to the different growth conditions[99], it is assumed that biogas production yield will be affected by *T. variabilis* grown in wastewaters prior to anaerobic digestion, through expression of various proteins. However, no research has yet demonstrated the effect of these generated macromolecules, which consequently impact bio-degradability of biomass during hydrolysis. Also, more investigation is required to be undertaken on the effect of co-digestion of *T. variabilis* and operational conditions such as hydraulic retention time and proportion of inoculum and substrates[100] as the main parameters known in bio-methane production. The results of previous studies indicated that variety and abundance of acetate oxidizing syntrophic bacteria significantly influence conversion efficiency of *T. variabilis* to bio-methane. Improving biogas conversion yield would be an interesting subject particularly in industrial wastewater treatment plants for coupling bioremediation with methane production.

The most common form of bioenergy generated from *T. variabilis* is hydrogen, a clean source of fuel that can be used either directly in internal combustion engines or in fuel cells to generate electricity[101]. The environmental benefits offered by renewable hydrogen are clear, particularly if the energy is being harnessed from microorganisms. Different species of algae[102] have been identified as potential sources of bio-hydrogen. Basic research in this field started with investigating bacterial hydrogen production in

the 1920s and later in the 1940s, attention shifted to microalgal hydrogen production[103]. Development in applied research started in the early 1970s[103]. Across several species used as cyanobacterial sources of bio-hydrogen[104], *T. variabilis* has attracted much attention owing to its 2-fold benefits in bio-hydrogen production and its considerable potential in nutrient removal from wastewater[9-11]. Table 2 presents hydrogen production yield of *T. variabilis* under different experimental conditions. Since this species has been widely investigated for hydrogen generation, here we focus on the biochemistry, cultivation methods, and utilization of wildtype and mutant strains for phycoremediation and/or fuel production.

6.3 Biochemistry and genetics of bio-hydrogen generation using *T. variabilis*

Algal hydrogen production occurs via light-dependent and light-independent reactions. Several articles have reviewed light- and dark-driven bio-hydrogen production[105-108]. The former includes bio-photolysis and photo-fermentation, which utilise sunlight, water, and CO_2, as energy, electron, and carbon sources, respectively. Light-independent hydrogen generation includes dark fermentation, which uses carbohydrate substrates, such as found in organic waste materials. Light-dependent processes are mainly driven by photosynthesis[109]. Hydrogen can be generated aerobically as a product of oxygenic photosynthesis or under anaerobic conditions, as a by-product of the conversion of organic substrates to acidogenic materials[106, 110]. Here, we have reviewed H_2 production methods using *T. variabilis* with specific focus on the biochemistry of enzyme systems.

The complete genome of *T. variabilis* ATCC 29413 was sequenced by the department of energy (DOE) Joint Genome Institute (JGI)[38] and deposited in the European Molecular Biology Laboratory (EMBL)/GenBank/ DNA Data Bank of Japan (DDBJ) database[38]. The genome size is 7.1 Mbp, with a total of ~5754 predicted protein-coding open-reading frames[38]. Availability of the full genome sequence should lead to a rapid increase in our knowledge of functional genes involved in growth, development, and metabolic processes in this organism. Thus, key gene networks supporting the different metabolic processes in various cell types are now within reach for identification.

Two enzyme systems of hydrogenase and nitrogenase play a major role in algal hydrogen production[106, 111]. *T. variabilis* hydrogenase and nitrogenase enzymes and genes encoding these proteins have been the subject of much research[112]. Hydrogenase enzymes play the main role in photolysis-dependent hydrogen production as well as hydrogen utilisation. There are two kinds of hydrogenase enzymes: (i) S-Fe hydrogenase (known as reversible or soluble hydrogenase), encoded by *hoxEFUYH* genes[113-115], function in generating molecular hydrogen to reduce NAD required for CO_2 absorption in the ribulose bisphosphate cycle and (ii) Ni- Fe hydrogenase (known as uptake or membrane hydrogenase), encoded by *hupSL* genes[116], which catalyses electron transfer from molecular hydrogen to the respiratory chain. Theoretically, efficiency of H_2 production in direct photolysis is high (40.1%) but it is not practically feasible due to the high inhibitory effects of O_2 on the reversible hydrogenase[103, 106, 117].

Since nitrogenase enzymes are sensitive to oxygen, this requires nitrogenase-catalysed H_2-generating reactions to be separated from oxygen-containing environments. Therefore, indirect photolysis evolved, in which hydrogen is generated in two separate stages. In the first stage, electrons derived from water splitting are consumed in CO_2 fixation to sugars. Thus, CO_2 is stored in vegetative cells as

carbohydrate and then transported to heterocysts, where the sugar is used as an electron donor[118, 119]. In the second stage, breakdown of the sugar releases electrons used to produce hydrogen by nitrogenase enzymes[103]. In the heterocysts of *T. variabilis*, groups of *nif* and *vnf* genes are expressed to give rise to metal ion-dependent nitrogenase, which is responsible for nitrogen fixing and generates hydrogen as a by-product of the process. There are three main gene clusters encoding nitrogenases, depending on distinct prosthetic groups: Mo-Fe (*nif*1 and *nif*2 genes), V-Fe (*vnf* genes) and Fe-Fe (*anf* genes) nitrogenases. Mo-Fe nitrogenases are encoded by (i) groups of *nif*1 genes, including *nifBSUHDKENXW*[20, 120-123] and three open reading frames expressed under diazotrophic conditions, and (ii) *nif*2 genes expressed under anoxic conditions. The latter are essentially the same (*nifBSUHDKENXW*) genes with the exception of a few differences, such as the fusion of *nifX* and *nifEN* genes into a single open reading frame[124]. V-Fe nitrogenase contains $\alpha\beta\delta$-subunits and scaffold proteins encoded by *vnfDKGENH*[125] which is expressed under diazotrophic conditions in the absence of Mo, with or without V. The third nitrogenase known as Fe-Fe nitrogenase is encoded by *anf*HDGK[126]. Further reviews and reports of *T. variabilis* nitroganase gene expression and heterocyst metabolism have been published[124, 125, 127-131]. Although indirect photolysis is more feasible than direct photolysis, the H_2 production efficiency is lower (16.3%) due to the multiphase process and ATP consumption required to drive the nitrogenase activity[103].

Other options based on fermentation of biomass have been developed. Hey *et al*[132] reviewed key factors affecting photo and dark fermentation, including substrates, inoculum density, and environmental conditions. Light fermentation is similar to indirect photolysis. During indirect photolysis, endogenous organic compounds are used to produce hydrogen, while in photofermentation exogenous organic compounds are consumed for use in generating electrons. Thus, fermentation is considered the most promising method for microbial H_2 production[103]. Unlike light driven processes, during dark fermentation organic substrates are used both as energy and electron sources. Bartacek *et al*[133] summarized fundamentals of fermentative H_2 generation in different microorganisms. Although anaerobic bacteria are mostly used in dark fermentation, it was reported that the combination of photo and dark fermentation of photosynthetic bacteria and algae could significantly enhance the bio-hydrogen productivity[134]. To the best of our knowledge, there are no reports on application of *T. variabilis* in such a hybrid system. Thus, further research on metabolic pathways of dark-driven hydrogen generation using *T. variabilis* is required.

6.4 Factors affecting H_2 generation in *T. variabilis*

Maximal H_2 production from algal cultures can be achieved through optimisation of key physicochemical factors with a regulatory influence on the generation capacity. High light intensity inhibits H_2 production rate due to up-regulation of photosynthesis, which enhances oxygen accumulation and consequent inhibition of nitrogenases[104, 135]. Darkness, on the other hand, can block H_2 production via depletion of carbohydrate reserves, which triggers a deficiency of energy and electrons needed for nitrogenase activity[118, 136]. The negative effects of excessive oxygen can be overcome through degassing or generating a partial vacuum[137], sparging with argon gas[138] and temporal separation of the photosynthetic phase from the H_2 generation stage[48]. Based on the 2-stage system for H_2 production, a flat panel photo-bioreactor was found to increase biomass production in the first stage with N-replete medium, and the H_2 generating in

the second stage with N-depleted medium sparging with pure Ar[48].

The presence of a nitrogen source in the growth medium severely decreases H_2 production, particularly when the nitrogenase enzyme machinery is the dominant H_2-producing complex. This is because inorganic nitrogen suppresses differentiation of heterocysts, thereby removing heterocyst-dependent generation. Although H_2 could be generated as a by-product of N-fixation utilizing atmospheric nitrogen as noted earlier (section 5.3) and the frequency of heterocysts increases in N-free growth medium[139], the innate biochemical inefficiencies of this process reduce H_2 gas production. About 75% of electrons are diverted away from H_2 synthesis to ammonium reduction, and this loss can be averted by the absence of atmospheric nitrogen[118].

H_2 production rate diminishes with decreasing pH[140]. A study focusing on pH effects demonstrated that algal growth rate was 3 times higher in the presence of Mo- or V-nitrogenase than in the presence of Fe- nitrogenase and maximum H_2 production was attained in the presence of V-nitrogenase in a wide range of alkaline cultures (pH 7-9)[141]. Mo and V are required as essential components in Mo-nitrogenase and V-nitrogenase based H_2 production, respectively, provided in BG and AA growth media[118, 142]. Although Mo significantly increases H_2 production rate, it has a specific upper limit (1.6 mM) beyond which further increases do not influence productivity[118]. The maximum light to hydrogen energy conversion efficiencies were achieved in Allen-Arnon media, where a larger heterocyst frequency was noted[48].Temperature is another parameter affecting H_2 photo-production. A short-term thermal stress (30-36 ^oC) enhances nitrogenase activity of *T. variabilis*[143]. The results of studying CO_2 concentration, as a carbon source, demonstrated that an optimum initial CO_2 molar fraction of 0.05 is required for maximum biomass production[56]. Additionally, inclusion of a carbon source, such as glucose, gives rise to further hydrogen production[118].

6.5 Genetic approach for increasing *T. variabilis* H_2 production

Manipulation of the parameters dealt with in the preceding section (e.g., pH, temperature, and nutrients) can be used to increase hydrogen production by algal systems. However, substantial research efforts have been directed at other key targets to enhance hydrogen yield. For example, the reversible hydrogenase enzyme system, which can consume as well as generate hydrogen, is an obvious target for inactivation using genetic approaches. Similarly, the uptake hydrogenase, which only consumes H_2 can be targeted for gene deletion. Accordingly, a *T. variabilis* loss-of-function mutant (AVM13) in the *hupSL* gene, which encodes the uptake hydrogenase, had at least a 3-fold increase in H_2 production when compared to the wildtype[144]. Nitroso-guanidine mutagenesis was used to generate PK84 and PK17R *T. variabilis* mutants with loss-of-function mutations impairing uptake hydrogenase in both mutants and an additional impairment of bidirectional (reversible) hydrogenase in PK84[145]. When compared to the wildtype, H_2 production in PK17R and PK84 was increased 1.4-fold and 4.3-fold, respectively[146]. Notably, while wild-type strains produce appreciable amounts of H_2 only in an argon atmosphere (to preclude O_2), the mutant PK84 produced H_2 in a CO_2 atmosphere, which is more desirable from a process scale-up perspective[147]. While mutants are showing great promise (Table 2), environmental concerns about the release of genetically modified organisms exist[148]. Since open biomass production systems are likely to be used in combined phycoremediation and bioenergy generation, use of mutant algae is inevitably problematic.

6.6 Feasibility of integrated biofuel production and wastewater treatment

In order to make the application of microalgae system feasible at industrial scale, it is vital to use sunlight as an energy source to significantly reduce the operation costs, relative to using artificial lighting. The mutant form of *T. variabilis* has demonstrated viable growth in ambient outdoor conditions[149-151]. It was shown that in such conditions, this species can prolong hydrogen generation without medium refreshment for 40 days[151]. Also the feasibility of growing the wildtype of this species in wastewater had promising results[9]. Although there are some pilot studies concentrated on bioenergy production and/or wastewater treatment using *T. variabilis*, there still is lack of specific research examining performance at larger industrial scale. However, several research groups have demonstrated that under certain circumstances, large-scale H_2-photo production using microalgae is viable[152]. Previously, scaling up the algal systems for bioenergy production was investigated from different techno-economic and socio-environmental angles considering various parameters, such as water and energy consumption, cultivation and harvesting technologies, and associated costs and savings[84]. Open raceway ponds with lower capital and operational costs, in comparison to PBRs, represent an attractive option for commercial large-scale bioenergy production[153]. It was demonstrated that this technology provides a "win-win strategy"[154], having remarkable productivity with an average capacity of 40-70 tons biomass.ha^{-1}.year^{-1} during bioremediation process, which can be coupled with biorefinery[153]. Results of a feasibility study for integration of wastewater treatment and biogas production using microalgae systems indicated positive benefits on payback period and internal rate of return (*IRR*) for biofuel production when considering environmental revenue and cost–savings, such as bio-products income, carbon credits and wastewater treatment[155]. The same strategy was reported as a feasible solution for using algae and bacteria cocultures to couple bioremediation and biofuel production [156]. Economic feasibility of commercial raceway pond use in wastewater treatment and biogas production should consider the pond size, electricity requirements, and thermal capacity for bio-methane production[157]. In such a system, the required CO_2 and thermal energy could be met by generation via combined heat and power production (305 kg CO_2.d^{-1} and 488 kWh.d^{-1}) coupled with biogas production system[157]. Therefore, the limited information on feasibility of bioenergy production using microalgal systems points to a beneficial outcome with significantly reduced energy consumption and costs if the system integrates with bioremediation to cover nutrient (40−100 kg of N and 3-12 kg of P) and water (11−13 ML.ha^{-1}.year^{-1}) requirements[153].

7 Challenges and opportunities

There are several studies on various aspects of *T. variabilis* biology, cultivation, and application to phycoremediation. Here, we have provided a coherent synthesis of the literature by presenting how surveying the interrelation between different studies could develop a route to industrial application of *T. variabilis* in bioremediation and production of sustainable bioenergy, discussing challenges, opportunities and research gaps as bellow:

1) Bioremediation performance of *T. variabilis* illustrated promising results in bioabsorption of nutrients and heavy metals such as Cu, Zn, and Ni. Remediation of different metals may influence gene expression and enzymatic function. This would be more important when the biomass harvested from remediation process

is used directly for bioenergy production. Thus, research into the effects of remediation on metabolic pathways, which control bioenergy generation, in this species needs to be undertaken.

2) There are some pilot studies showing the utility of *T. variabilis* in bioremediation using artificially constituted "wastewaters". However, actual wastewaters may contain unknown hazardous compounds and allelochemicals, which can negatively influence algal growth. For example, $nTiO_2$, Microcystins, Harmane and FischerellinB restrict application of algae in wastewater streams containing such pollutants. Therefore, it is necessary to examine actual biomass generation in different real wastewater streams for scaling up phycoremediation at industrial scale for biorefining purposes.
3) The application of *T. variabilis* for biodiesel and bio-ethanol production has not been widely studied. Although *T. variabilis* is not considered an oleaginous species, environmental stress can induce cells to produce significant amounts of lipid. The molecular genetic basis for the enhanced lipid synthesis requires further research. Also, bio-ethanol production using this organism is not viable due to the challenge of polysaccharide loss together with associated costly and high energy-consuming technologies required for the process. Therefore, more efficient solutions are required.
4) Biogas generation using this alga is promising, particularly when coupled to wastewater treatment. In order to improve bio-methane yield, first it is necessary to evaluate the effect of the bioremediation process on algal cell composition, protein profile and biomass bio-degradability. This would provide valuable information to boost the initial hydrolysis processes via applying the most appropriate pretreatment technology. Also, applying co-digestion technologies can enhance anaerobic digestion kinetics and, from an economic aspect, there are still several improvement methods such as optimizing the proportions of microalgal cells for declining the anaerobic digestion hydraulic retention times which need further investigation.
5) Bio-hydrogen production using *T. variavilis* is focused on light-driven methods and the potential of using this species in dark-driven hybrid systems, particularly in combination with dark fermentation, has not yet been surveyed. Although the challenge of oxygen inhibitory effects on hydrogen generation can be averted by degassing, partial vacuum, sparging Ar, applying two-staged H_2 generation method or utilysing mutants of *T. variabilis*, presented solutions are costly and carry environmental risks. Therefore, still further research is necessary to develop cost-effective and environmentally friendly alternatives for robust bio-hydrogen production systems.

8 Future prospects

In this review, we have sought to collate the wide literature on *Trichormus variabilis*, bringing data together from many different aspects, including morphology focusing on different types of cell differentiation and physiology, together with a review of growth parameters and conditions where the species may be used in remediation of wastewaters coupled with production of different forms of liquid and gaseous bioenergy, such as biodiesel, bio-ethanol, bio-methane and bio-hydrogen. We attempted to distil the key information available on *T. variabilis* on phycoremediation and bioenergy production to enable further research on optimization of robust technologies for supplying clean water

and sustainable energy resources. Trends in research of *T .variabilis* during the last decades has focused on the main aspects of (i) biology, (ii) nutrient removal and (iii) bioenergy production, and considered the effects of several stress factors on growth and lipid production. There is a promising path for future application of this species even though there are still major environmental and economic issues which need to be resolved to optimise *T. variabilis* for industrial usage. The valuable potential of this microorganism arising from the differentiation of vegetative cells into other cell types for adaptive responses to different environmental conditions provides a great opportunity to develop platforms for commercial exploitation of this species. It is important to elucidate the mechanisms of cell differentiation and metabolic changes associated with exposure to stress.

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Competing interests

The authors have no competing interests.

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Table 1. Bioremediation potential using *T. variabilis*.

Experimental conditions	Removed compounds	Efficiency (%)	Ref
Preliminary growth: MBL medium, 25–35 °C, day light for 14 days. **Treatment process:** Mixed domestic-industrial wastewater, 25–35 °C, daylight.	BOD	89.3	[9]
	COD	52.6	
	TSS	44.6	
	TDS	38.8	
	Fats, Oil and Grease	68.2	
	Zn	81.2	
	Cu	91	
Preliminary growth: BG_{11} medium, 30 °C for 15 days. **Treatment process:** ~16% of BG_{11} medium mixed with textile industry effluent wastewater for 25 days, 30 °C, aerobic condition.	BOD	83	[10]
	COD	75	
	SO4	55.4	
	Ni	63	
	Zn	67	
	Ca	17.5	
	Mg	28	
Preliminary growth: MDM medium, 27.5 °C, ~ 65 $\mu mol.m^{-2}.s^{-1}$, aerated containing 1% CO_2 for 7 days. **Treatment process:** using supplemented medium, phenols concentration 40 µM, 25 °C for 120 h.	*o*-Nitrophenol	100	[71]
	m-Nitrophenol	100	
	p-Nitrophenol	4	
	2,4-Dinitrophenol	95	
	2,4,6-Trinitrophenol	51	
	Bisphenol A	23	
Preliminary growth: Fogg medium, 24 ± 1 °C, ~ 55$\mu mol.m^{-2}.s^{-1}$, 16 h light / 8 h dark cycle for 22 days. **Treatment process:** supplemented medium, Standard stock solution of Zn^{2+} ions (1000 ± 2 $mg.L^{-1}$)	Zn	85.1	[158]

Preliminary growth: calcium alginate immobilized cell **Treatment process:** supplemented medium, 11-10 ppm concentration of lead	Pb	96	[159]
Preliminary growth: Chu-10 medium, 28 ± 2 ºC, daylight ~ 45 µmol m^{-2} s^{-1} **Treatment process:** supplemented medium, Stock solutions of chromium concentrations of (10 - 100 mM) prepared by dissolving $K_2Cr_2O_7$.	Cr	54	[160]
Preliminary growth: Allen and Arnon medium, 25 ºC, 15 µmol.m^{-2}.s^{-1} **Treatment process:** Ammonium ions were added in concentration of 0.5 mg.L^{-1}, immobilized cells in the photobioreactor for 25 days.	Ammonium	90	[13]

Table 2. Hydrogen production yield using *T.variabilis* under different experimental conditions.

Strain (wild/mutant)	Experimental conditions	Medium	Hydrogen production rate	Ref
ATCC 29413	Growth: 30 °C, 60 μmol.m^{-2}.s^{-1} H_2: 5% CO_2, Ar (25 ml min^{-1}), 35 °C, 70 μmol.m^{-2}.s^{-1}, (a) 1.6 mM Mo, (b) 10 mM glucose	BG_0	(a) 44 μmol.mg $chla^{-1}$.h^{-1} (b) 49 μmol.mg $chla^{-1}$.h^{-1}	118
	Growth: 95% air + 5% CO_2, 30 °C, 65 μmol.m^{-2}.s^{-1} H_2: Ar (45 ml min^{-1}), 30 °C, 150 μmol.m^{-2}.s^{-1}	BG_0	0.9 mL.g dry $cell^{-1}$.h^{-1}	48
	Growth: 30 °C, 35-161 μmol.m^{-2}.s^{-1}, 190 rpm H_2: 100% Ar	BG_{11} AA BG_{11}	1 mL.g dry $cell^{-1}$.h^{-1} 5.6 mL.g dry $cell^{-1}$.h^{-1} ~6 mL.g dry $cell^{-1}$.h^{-1}	41
	Growth: Ambient air, 25 °C , 5 μmol.m^{-2}.s^{-1} (bottom), 15 μmol.m^{-2}.s^{-1} (surface) H_2: 100% Ar, Hydrophilic cuprammonium rayon hollow fibers PBR, medium was heated at 50°C prior injection	AA	17- 20 mg.g dry $cell^{-1}$.h^{-1}	13
	Growth: ~ 67.5 μmol.m^{-2}.s^{-1} H_2: Ar + 5% CO_2, 77 mM Tween 85	AA	0.44 ± 0.03 mL.mg dry $cell^{-1}$.h^{-1}	161
CCAP 403/4B	Growth: 1.7% CO_2, 28 °C, continues 45-55 μmol.m^{-2}.s^{-1}, 140 rpm H_2: Vacuum degassed (270-300 torr),170-180 μmol.m^{-2}.s^{-1} for 5 hr	AA_0	12 .5 mL.g dry $cell^{-1}$.h^{-1}	140
	Growth: Air without CO_2, 28 °C, 15 μmol.m^{-2}.s^{-1}, 140 rpm H_2: No gas phase, cells immobilized on hollow fibers; 25 μmol.m^{-2}.s^{-1} (surface), 13 μmol.m^{-2}.s^{-1} (bottom), medium was heated at 50 °C prior injection	AA_0	20 mL.g dry $cell^{-1}$.h^{-1}	137

PK84	Growth: Air + 2% CO_2(500 mL.min^{-1}), 12 h light (36 °C)/ 12 h dark (14- 30 °C) H_2: (a) air, (b) air+ 2% CO_2, (c) 80% Ar + 20% O_2, (d) 100% Ar, 332 µmol.m^{-2}.s^{-1}	AA_0	(a) 106.0 ± 7.0 µmol.mg chla^{-1}.h^{-1} (b) 81.0 ± 10.0 µmol.mg chla^{-1}.h^{-1} (c) 190.0 ± 8.0 µmol.mg chla^{-1}.h^{-1} (d) 191.0 ± 11.0 µmol.mg chla^{-1}.h^{-1}	149
	Growth: Air+ 2% CO_2 (500 mL.min^{-1}) H_2: Outdoor PBR, ~ 1.84 mol.m^{-2}.s^{-1}	AA_0	80 mL.h^{-1}.PBR v^{-1}	150
	Growth: air + 2% CO_2 (500 mL.min^{-1}), 30 ^{0}C H_2: Outdoor PBR, sun light	AA_0	60-140 mL.h^{-1}.PBR v^{-1}	151
(a) ATCC29413 **(b) PK84**	Growth: air + 2% CO_2 (0.5 L.min^{-1}), 30 ^{0}C, 113 µmol.m^{-2}.s^{-1} H_2: 100% Ar, 30 ^{0}C, 200 µmol.m^{-2}.s^{-1}	AA	(a) 39.4 nmol.µg chla^{-1}.h^{-1} (b) 32.3 nmol.µg chla^{-1}.h^{-1}	135
(a) ATCC29413 **(b) PK84** **(c) PK17R**	Growth: 25% N_2, 2% CO_2, 73% Ar (250 mL.min^{-1}), 90 µmol.m^{-2}.s^{-1} H_2: Ar, 30 ^{0}C, 140 µmol.m^{-2}.s^{-1}, N/C starvation	AA_0	(a) 1.62- 3.07 nmol.µg prot^{-1}.h^{-1} (b) 6.91-12.6 nmol.µg prot^{-1}.h^{-1} (c) 2.24-4.10 nmol.µg prot^{-1}.h^{-1}	146
AVM13	Growth: Air, 1% CO_2, 30 ^{0}C, 100 µmol.m^{-2}.s^{-1} H_2: Washed with N-free medium	BG_0	68 nmol.µg^{-1}.chl *a*$^{-1}$.h^{-1}	144